Response: Characteristics of IL-17-Producing γδ T Cells

Caroline E. Sutton,[1,3] Stephen J. Lalor,[1,3] Cheryl M. Sweeney,[1] Corinna F. Brereton,[1] Ed C. Lavelle,[2] and Kingston H.G. Mills[1,*]
[1]Immune Regulation Research Group
[2]Adjuvant Research Group
School of Biochemistry and Immunology, Trinity College, Dublin 2, Ireland
[3]These authors contributed equally to this work
*Correspondence: kingston.mills@tcd.ie
DOI 10.1016/j.immuni.2010.01.007

We are pleased to read that our paper (Sutton et al., 2009), as well as that of Martin et al. (2009), has stimulated a debate on the importance of γδ T cells in interleukin-17 (IL-17) production and the signals that may drive this production. We acknowledge the contribution that O'Brien and colleagues have made to our understanding of the role of IL-17 production by γδ T cells in autoimmunity in our manuscript. However, we do not agree with their assertion that we "claim priority" or that we did not acknowledge previous contributions to the discovery that "γδ T cells can produce IL-17 without deliberate stimulation via the T cell receptor." Indeed we cited the contribution by Shibata et al. (2007) and state in the discussion section of our paper: "It has also been reported that stimulation of peritoneal exudate cells with IL-23, but not by IL-6 and TGF-β, induced IL-17 production by γδ T cells (Shibata et al., 2007). Our study demonstrates a requirement for IL-1 and shows that IL-23 and IL-1β promote innate IL-17 production from γδ T cells and provides an alternative mechanism whereby IL-23 and IL-1β may contribute to host defense and autoimmune inflammation."

We did not cite the paper by Cheng et al. (2008) because, like the data in Figure 8 of Shibata et al. (2007), it was not based on IL-17 production by γδ T cells from naive mice, but from mice immunized with uveitogenic peptide in CFA or infected with *E. coli*, respectively. Therefore, as alluded to by O'Brien et al. (2010) in their Letter, it is possible that these γδ T cells have recently been activated through the TCR in vivo. The study by Shibata et al. (2007) did examine IL-17 production by γδ T cells from naive mice (Figure 7 in their paper), but used unpurified peritoneal exudate cells (PECs) and showed, by intracellular cytokine staining (ICS), that 50% of γδ T cells within the PEC population secrete IL-17 in response to in vitro stimulation with IL-23. However, it is not possible from this approach to conclude that IL-23 acted directly on the γδ T cells because they did not examine IL-23R expression on γδ T cells. Furthermore, with whole PEC populations, which include dentritic cells (DCs) and macrophages, it is not possible to rule out the contribution of IL-1 or other cytokines, expressed by macrophages or DCs, either constitutively or in response to TLR agonists, which are present in many FCS preparations. Finally, a problem with the ICS data, which is a general problem with the vast majority of data generated with this approach, is that the cells were restimulated with PMA and ionomycin after stimulation with IL-23 and the findings need to be confirmed with approaches that do not require secondary stimulation in vitro.

There are a number of very important differences from the previous work and advances made by our study over those reported by Shibata et al. (2007) and by Cheng et al. (2008). Our study provides unequivocal evidence based on cells from gene-targeted mice, as well as in vitro depletion and purification approaches, with assays based on PCR, ELISA, and ICS, that γδ T cells from naive mice express IL-17A, IL-17F, IL-21, IL-22, IL-23R, and RORγt in response to IL-1 and IL-23 in the absence of stimulation through the TCR. In particular, our report (Sutton et al., 2009) together with our earlier paper (Sutton et al., 2006) have highlighted the importance of IL-1 and its synergy with IL-23 in providing the signals to promote IL-17 production by T cells both in vitro and in vivo.

O'Brien et al. (2010) suggest that γδ T cells may require prior TCR stimulation to produce IL-17 and propose that the responding γδ T cells in our system "may have already encountered a ligand, and would not have responded in vitro to cytokines only were it not for that." We did assess IL-17 production by γδ T cells from unimmunzed mice, in addition to γδ T cells from mice immunized with MOG and CFA (to induce EAE). Our data in Figure 3 clearly show that γδ T cells purified from spleens of unimmunized mice do not secrete IL-17 or express IL-17 mRNA in vitro in the absence of IL-1 and IL-23 stimulation. Although we cannot rule out a role for MTB-derived ligands in TCR-mediated activation of γδ T cells in mice with EAE, this cannot account for the IL-17 production we observed in γδ T cells from naive mice after stimulation with IL-1 and IL-23 without TCR engagement. However, we do not discount a role for stress-induced host molecules in activation of γδ T cells in vivo. Indeed, using ICS, which involved in vitro stimulation of the cells with PMA and ionomycin, we did observe IL-17 production by γδ T cells from lymph nodes of naive mice, which was enhanced when mice had been injected with IL-1 and IL-23 (Sutton et al., 2009, Figure S2B). Therefore, it is possible that endogenous TCR ligands may activate γδ T cells in vivo in naive mice; however, we have no evidence to support this hypothesis. Jensen et al. (2008) also found that a large fraction of γδ T cells from naive mice secreted IL-17 (detected by ICS after in vitro stimulation with anti-CD3), and this was enhanced 3–4 days after immunization with MOG and CFA, but interestingly they reported that γδ T cells that encounter ligand during development produced IFN-γ, whereas antigen-naive γδ T cells secreted IL-17. These findings are consistent with our conclusion and,

those highlighted in the *Immunity* preview by Kapsenberg (2009), that γδ T cells are important innate source of IL-17 early in immune responses in vivo.

O'Brien and colleagues maintain that we "run into difficulties when they attempt to determine which subset of γδ T cells is responding" and also point to confusion in the terminology used to describe γδ T cell subsets. We accept that the terminology is confusing, but this is not of our making. It arises from the use of two independent TCR nomenclature systems for γδ T cell subsets, that of Raulet (Garman et al., 1986) and that of Tonegawa (Heilig and Tonegawa, 1986). We followed the Tonegawa nomenclature (Heilig and Tonegawa, 1986) throughout, which is the same as that used by O'Brien and colleagues. There is no confusion about Vγ5 versus Vγ7, the antibody we used was specific for Vγ5 (mAb clone 536). Our rationale for testing only a selection of Vγ and Vδ subsets was partly based on commercial availability of antibodies, but also the need to examine a combination of γδ T cell subtypes that may or may not be expected to produce IL-17 or infiltrate the CNS. Regardless of our rationale for choosing which subset to study, our data clearly shows that Vγ4 are a critical γδ T cell subset producing IL-17 during development of EAE.

ACKNOWLEDGMENTS

K.H.G.M. is a cofounder, minority shareholder, and member of the scientific advisory board of Opsona Therapeutics, a university start-up company involved in the development of anti-inflammation therapeutics.

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